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NOVEL ALLELES OF *Medicago truncatula* AUTOREGULATION OF NODULATION MUTANT

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ABSTRACT

Two new alleles of *Medicago truncatula*-Autoregulation of Nodulation (AON) mutant *sunn* exhibited hyper-nodulation and shortened roots phenotype were discovered. The two mutant lines (NF2262&NF3306) contain tobacco Long Terminal Repeats (LTR) retro-transposon *Tnt1*-mobile element. *Tnt1*- insertion is precisely located at 462 bp and 406 bp upstream of ATG start codon in NF3306 and NF2262 respectively within *SUNN* gene. The two new independent mutant alleles relay under the *nitrogen-tolerant symbiosis* group (*nts*) similar to the previously described *sunn* mutant. This hyper-nodulating phenotype is resulting from defective in pathway of complex hormonal interaction. Since auxin and ethylene coordinated signals resulted in continuous root cells activity and elongation, different exogenous treatments were supplemented to growth media in attempts to elucidate the signals interaction complexity. While the minor concentration of Auxin 3- Indole Acetic Acid (IAA) enabled the two mutant lines to keep the shorter root length phenotype in comparing with R108, the higher concentration inhibited root growth in both mutant lines and R108. Data showed that the suppression ability of synthetic auxin transport inhibitor *N*-(1-naphthyl) phthalamic acid (NPA) had more negative influence on root elongation than IAA and it was notably that NPA strongly affects wild type than the two mutants. In the other hand, results indicated that root growth of *sunn* mutant is completely unaffected by Ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) in contrast with R108 which was strongly inhibited by adding 10µM of ACC than 0.1µM of ACC.

KEYWORDS: Leucine-Rich Repeat (LRR) Receptor Kinase, Super-Nodulation, *Sunn*, Phytohormones, Root Elongation, Auxin Transport

INTRODUCTION

Legume-rhizobia symbiotic interaction is considering as one of the most successful symbiosis. Such symbiosis establishment enables the plant to grow in nitrogen-poor condition. Inside the symbiotic organ which is termed nodule, rhizobia fix atmospheric N2 into NH3, which in turn can supply the host plant with its nitrogen desires. In return, bacteria's requirements are supplied by the plant. Auto-regulation of nodulation is the feedback process by which the plant can suppress the formation of excessive number of nodules (d'Erfurth et al, 2003), it excludes both root and shoot communication through long distance transduction. The shoot is suggested to perceive the signal that generated and translocate upon rhizobia, however the shoot to root signal is predicted to inhibit the formation of new nodules. The *Medicago truncatula SUNN* gene encodes a CLV1-like leucine-rich repeat receptor kinase that regulates nodule number and root length. All autoregulation-defective legume mutants, such as *Lotus japonicas hypernodulation aberrant root formation* (*har1*) (Schauseret al. 1998;Wopereis et al. 2000;Kawaguchi et al. 2002; Nishimura et al 2002), clavier (Oka-Kira et al. 2005), too much love (Magori et al. 2009), *Glycine max autoregulation receptor kinase* (*nark*) (Carrollet al.

1985;Nishimura et al 2002;Searle et al. 2003), *Pisum sativum sympiosis* (*sym29*) (Sagan &Duc 1996) and *Medicago truncatula sunn* display a hypernodulating phenotype (Schnabel et al. 2005). Despite that *SUNN* is the ortholog of both *HAR1*and *NARK* based on synteny and the mutated phenotype, its closest nucleotide sequence is *Arapidopsis CLAVATA1* (*CLV1*). CLV1 is specifically expressed in shoot apical meristem (SAM) while all the legume genes are expressed in various organs. Unlike the mutation in *CLV1* loci which exhibits fascinated shoot, mutations in *HAR1* orthologous loci do not show any *clv1*-like shoot phenotypes. This indicates that legumes CLV1-like genes perform a particular role in the directive of nodule development but not in the SAM size regulation.This study is aiming to precisely locate the *Tnt*1 insertion within the two mutant lines NF2262&NF3447.

SUNN GENE DESCRIPTION

The 3.5 kb gene encode a 974 amino acid protein which is 75–85% identical to LjHAR1, GmNARK and PsSYM29 consisting of 21 extracellular LRRs, a short trans-membrane domain, and cytoplasmic serine/threonine kinase domain. The coding sequence is disturbed by a single intron in a location conserved among LjHAR1, GmNARK, PsSYM29, and various other receptor kinases. *SUNN* gene located in chromosome 4 at position 400 kb (Schnabel et al. 2005).

PREVIOUSLY DESCRIPED *Mtsunn* ALELES

Sunn-1 is Jemalong A17 line, EMS mutagenesis, the predicted Arg to Lys change at kinase domain XI position in the protein product of the *sunn*-1 allele is affecting the phosphorylation ability of the kinase (Hanks &Quinn 1991; Penmetsa et al. 2003). *sunn-2* and *sunn-4* are Jemalong J5 are gamma-irradiation mutagenesis. Ser to Arg change in the protein product of *sunn-2* allele occurs just before the last leucine rich repeat, while *sunn-4 allele* is a null mutant with a base deletion creating truncating protein shortly after the initial signal sequence (Sagan et al. 1995; Saganet al. 1998). *sunn-3* is an EMS mutagenized seeds deriving from L416, a transgenic A17 line harboringp*MtENOD11-gusA* construct (Charron et al. 2004).It contains a single C to T base change in the kinase domain creating stop codon, eliminating all kinase domain XI by truncating the last 52 amino acids from the protein and.

MATERIALS AND METHODS

Plant Material

Medicago truncatula mutant lines NF3306, NF2262 (Noble Foundation *Tnt*1 mutant collection) was derived from *M. truncatula* R108-1 ecotype (Tadege et al. 2008). Both mutant lines are identified as super-nodulation mutant through *Tnt1*-mutants screening in Noble Foundation. The two mutant lines contains tobacco LTR retrotransposon*Tnt1*-mobile element. This was carried out by *in vitro* transformation via *agrobacterium tumifaciens* (d'Erfurth et al, 2003). They investigated that *Tnt1* transposes was activated during in vitro transformation and the insertions are stable during *Medicago truncatula* life cycle.

Bacteria Strain

Sinorhizobium meliloti strains *Sm1021* was used in *M. truncatula* inoculation (Galibert et al. 2001). *Sm1021* was used to inoculate seven days-old *Medicago* seedlings belong to our two mutant lines NF3306& NF2262 and the R108 wild type phenotype. Seedlings were grown in BNM medium, which is nitrogen free media used for nodulation test to monitor nodule phenotype of different plants (Ehrhardt et al. 1992). The overcome nodules were followed up three weeks post inoculation and the nodule numbers were accounted for each plant.

Genetics

Allelism test was carried out by crossing the two mutant lines together to monitor the progeny's phenotype. This test was carried out to investigate the ability of the two mutant lines in complement each other. In case that all members of F1offspring show hyper-nodulation phenotype we can assume that the two mutant lines exhibit a mutation in the same gene.

Genomic DNA Extraction and PCR

DNA from ten plants of each mutant line and the R108 wild type were extracted using DNeasy Plant DNA mini kit, QIAGEN, according manufactory's instruction. The DNAs from each line were pooled together and used for PCR reactions. All PCR reactions were done using EmeraldAmp MAX PCR Master MIX, TAKARA, this taq is suitable for amplification long size fragments

Physiological Studies

Variations in phenotype between the two mutant lines and the control plant were elucidated under some differences of growth conditions. IAA, ACC, and Synthetic auxin transport inhibitor NPA was supplemented separately to BNM media and the root growth were monitored at different time intervals.

Statistical Analysis

All statistical analysis has been done using ASSISTAT Version 7.7 beta*t*-test

RESULTS

- **Mutant Phenotype**

NF3306 and NF2262 showed short root and hyper-nodulation phenotype when growing in BNM media comparing to the wild type phenotype R108. Nodules number was shown to be 60±5 per each plant belongs to our mutant lines while it shown to be 10±2 nodules per wild type plant. This phenotype strongly enables them to be either *sunn*, *sickle* or both of them.

- **Genetics and Allelism Test**

The crossing between the two mutated lines NF2262 and NF3306 proofed that the two lines did not complement each other in F1 progeny. F2 progeny resulting from self-pollination did not show any wild type phenotype. Consequently, the two mutant lines are allelic.

- **Molecular Characterization**

Molecular characterization depends on the presence of 610 bp Long Terminal Repeats (LTR) in both sides of the *Tnt1* transposon which is 5.3bp in length. The two oligonucleotide primers LTR4: 5'-TACCGTATCTCGGTGCTACA-3' and LTR6: 5'- GCTACCAACCAAACCAAGTCAA-'3 were used in mutant characterization using PCR technique (Ratet et al. 2006).

- **NF2262 and NF3306 Mutants Are Not Identified as Sickle Alleles**

DNA pool from each mutant line was subjected to PCR using different combination of oligonucleotide primers. One pair of oligonucleotide primer SIK-AF: 5'-ATGATGATATGATGTTTGATCATGAG-3' and SIK-ER: 5'-

TCACCTAAAAAAAAAGTATACATGC-3’ was designed and used separately with both LTR4 and LTR6 to scan the full length-*SICKLE gene*. PCR result reveals negative amplification for all tested combinations as R108 control, indicating that our mutant doesn’t belong to *Medicago truncatula sikle* mutant.

- **NF2262 and NF3306 Mutants Are Identified as *Sunn* Mutant Alleles**

One pair of Oligonucleotide primer SUNN3-F: 5’-CGCTGTTAGCTTCCATGCTT-3’ and SUNN3-R: 5’-ACGGTTACTCAATGTAGTTC-3’ was designed and used in combination with both LTR4 and LTR6 separately to screen the presence of *Tnt1* borders within *SUNN* gene. Although Oligos combinations, SUNN3-R &LTR4 and SUNN3-R&LTR6 failed to monitor any *Tnt1* border, SUNN3-F& (LTR4&LTR6 respectively) successes to amplify the *Tnt1* borders in both mutants line.The mutation of both lines located within extracellular LRRs domain of SUNN gene. In case of NF3306, *Tnt1*- insertion was mapped at 462 bp upstream ATG start codon of *SUNN* gene. This result was obtained by PCR using SUNN3-F&LTR6, giving rise to a 1475bp-fragment which was absent in NF2262 and wild type R108 (Figure 1A).

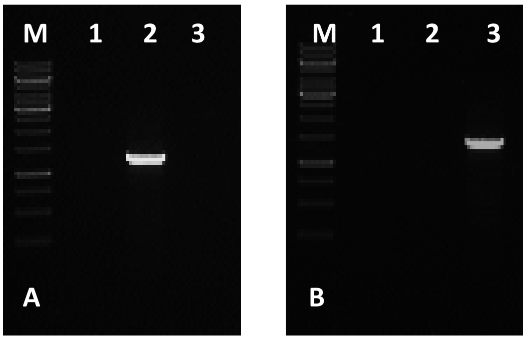

Figure1. (a) PCR Product Using SUNN3-F&LTR6: 1475 bp Band in NF3306 (2), Negative Result in NF2262 (3) and in Control R108 (1). (b) PCR Product Using SUNN3-F&LTR4: 1530 bp Band in NF2262 (3), Negative Result in NF3306 (2) and in Control R108 (1)

In case of NF2262, *Tnt1*-insertion was mapped at 406bp upstream ATG start codon of *SUNN* gene. This result was obtained by PCR using SUNN3-F&LTR4, giving rise to a 1530bp fragment that was absent in NF3306 and wild type R108 (Figure 1B). Insertion fragments were purified and sequenced to precisely map the *Tnt1* border within the two mutant lines. *Tnt1* insertion loci within NF2262 &NF3306 genomes are shown in (Figure 2). By this result we can authorize that both NF2262 and NF3306 are new alleles of *sunn* mutant.

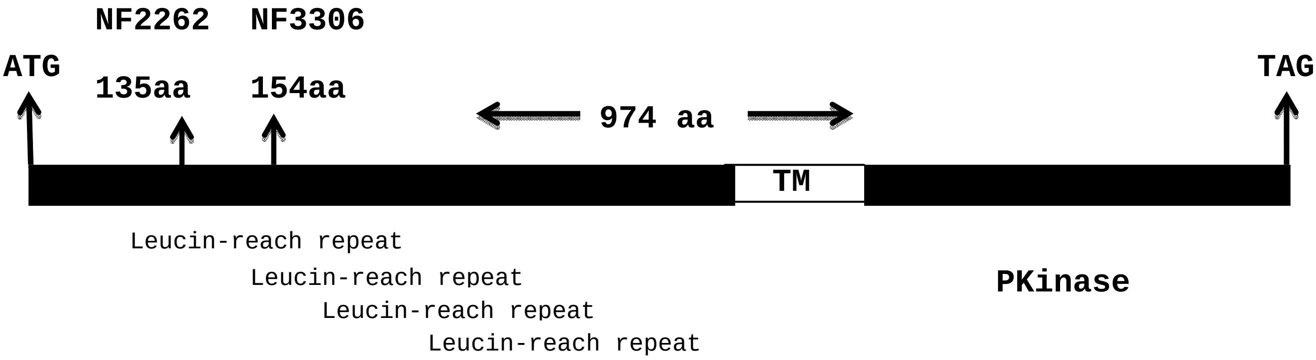

Figure 2: *Tnt1* Insertion is Located within LRR Domain of SUNN in Both NF2262 and NF3306

- **Physiological Characterization**

Nitrate Tolerant Phenotype

Both NF2262 &NF3306 showed nitrate tolerant phenotype, when inoculated with rhizobia under effect of 5 mM NH4NO3 in contrast with R108 wild type which failed to perform any nodule under the nitrate stress.

Phytohormones and Mutant Phenotype

Auxin and ethylene coordinated signals resulted in continuous root cells activity and elongation. In order to elucidate the defective signal within phyotohormones pathway, different concentration of exogenous hormones were added to growth media to compare the two *sunn* mutant allaeles with the wild type. The root lengths of wild type and the two mutant alleles (NF2262 and NF3306) were recorded at different intervals without any exogenous hormone treatment (Figure 3).

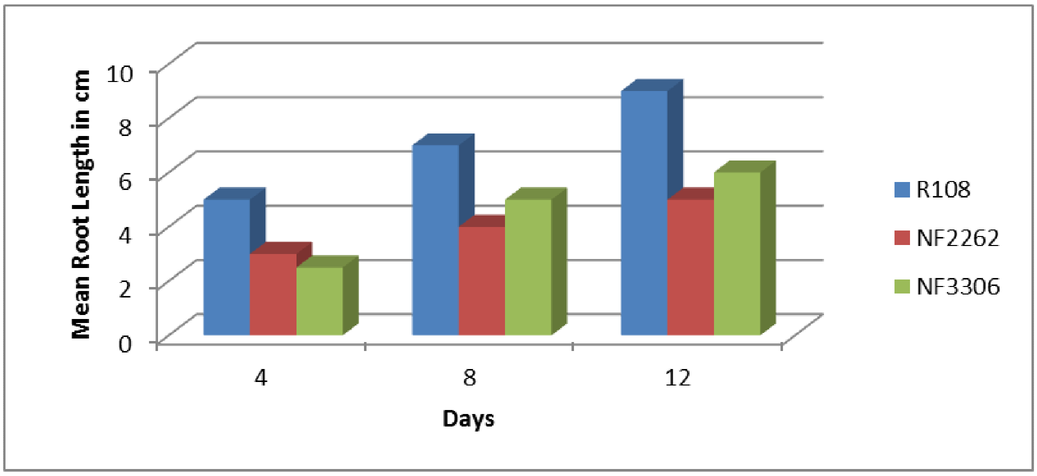

Figure 3: Comparison of Root Growth Rate between the Two Mutant Lines NF2262& NF3306 and R108 Wild Type without Hormonal Treatment at Different Time Intervals

Root Length Phenotyp in the Presence of Auxin, IAA and Auxin Inhibitor, NPA

Auxin is an important regulator of nodulation process, transported from the shoot to the root. Despite that the *sunn* mutant exhibited an increased amount of shoot to root auxin transport compared to the wild type, local auxin transport inhibition at the inoculation site occur in *sunn* as well as wild type (van Noorden et al. 2006). Penmetsa presented that auxin response gene GH3 expression is higher in *sunn* than in wild type prior to Rhizobia infection (Penmetsa et al. 2003). To test the effect of adding exogenous IAA in the rate of root growth, 0.1 µM&10µM of IAA was supplemented to growth media and the root lengths were scored every 4 days. While the minor concentration of IAA enabled the two mutant lines to keep the shorter root length phenotype in comparing with R108, the higher concentration 10µM IAA inhibited root growth in both mutant lines and R108. Figure (4) revealed that the inhibitory influence of 10µM IAA is strongly affected R108 than the two mutant lines. All values are significantly differentat $p < 0.01$ as shown in (Table 1).

Auxin transport can be repressed by auxin transport inhibitor, NPA. To test the effect of adding exogenous NPA in rate of root growth, 0.1 µM&10µM NPA was supplemented to growth media and the root lengths were scored every 4 days.

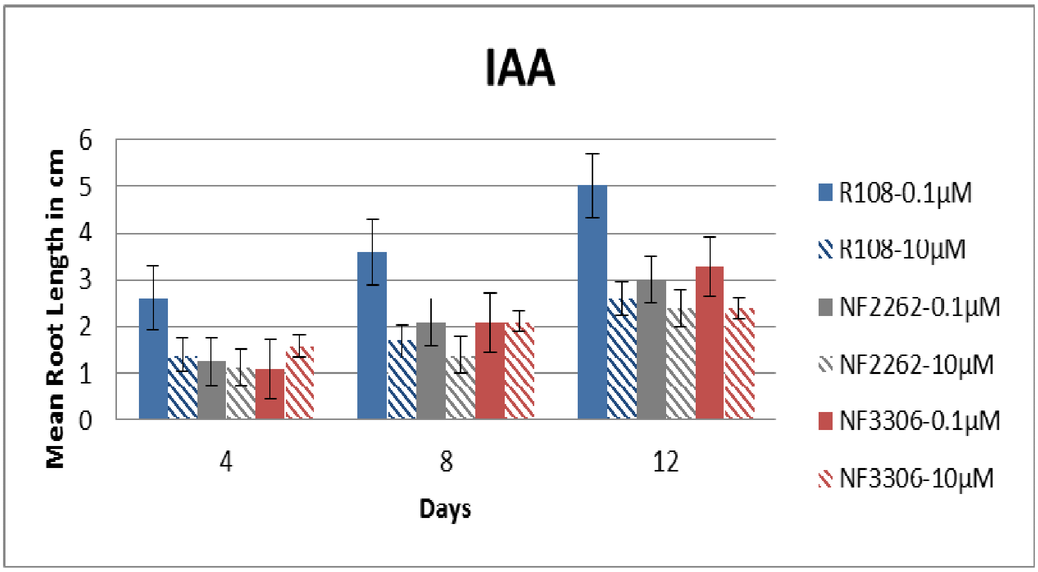

Figure 4: Comparison of Root Growth Rate between the Two Mutant Lines NF2262 & NF3306 and R108 Wild Type under Effect of Adding 0.1&10 µm IAA

Table 1: T Test for IAA Exp. at A Level of 5% Probability, Smd for Columns = 0.7187 Smd for Rows = 0.7187

GA = 2.25000 VC% = 25.34 Midpoint = 3.25000

	1µMIAA			10µMIAA		
	4 days	8 days	12 days	4 days	8 days	12 days
R108	2.6000 aC	3.6000 aB	5.0000 aA	1.4000 aD	1.7000 aD	2.6000 aC
NF2262	1.3000 bC	2.1000 bAB	2.8000 bA	1.0000 aC	1.4000aBC	2.2000 aA
NF3306	1.1000 bD	2.1000 bBC	3.5000 bA	1.6000 aCD	2.1000aBC	2.4000 aB

Results showed that the low and high concentrations have a negative impact in relative root growth in R108 and the mutant lines as well as shown in (Figure 5). Values are not significantly difference (Table 2). From previous results we can assume that root elongation suppression ability of NPA strongly affects wild type than the mutants and NPA has more negative impact on root elongation than IAA.

Root Length Phenotypes in Presence of Ethylene Precursor ACC

Ethylene is stimulating auxin biosynthesis and moderating auxin transport mechanism by modulation the transcription activity of several components involving in auxin transportation (Ru˚ zickaet al. 2007). To examine the influence of addition increasing concentration of ACC in the rate of root growth of the two mutant alleles; a concentration of 0.1 µM&10µM of ACC were added to the growth medium and the root length were scored every 4 days' time intervals and compared with those of wild type under the same condituion (Figure 6). Results indicated that root growth of *sunn* mutant is completely unaffected by ACC addition while wild type R108 root growth were inhibited with addition of 10µM of ACC than 0.1µM of ACC. Variance analysis showed that all values are significantly different at $p< .01$ as shown in (Table 3).

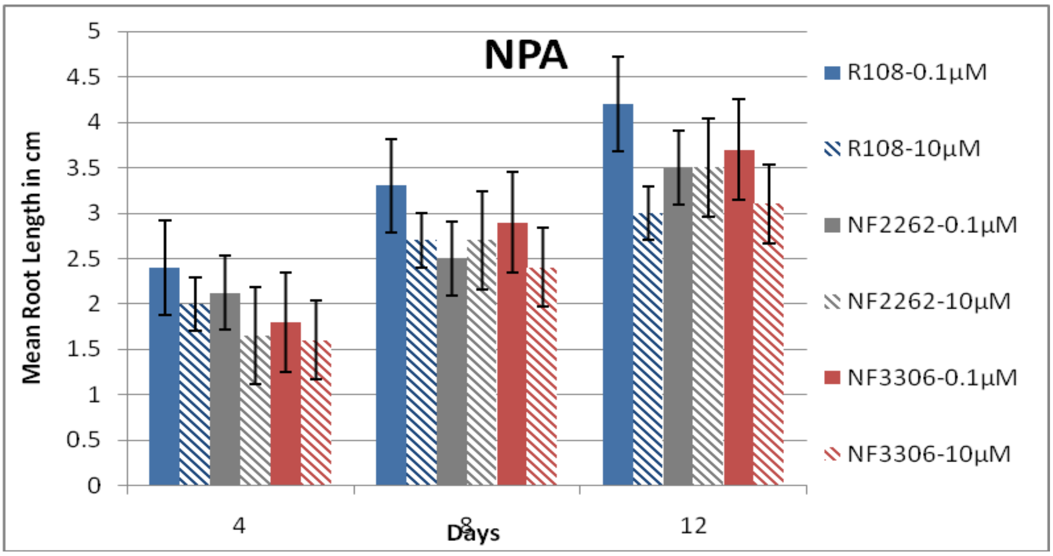

Figure 5: Comparison of Root Growth Rate between the Two Mutant Lines NF2262& NF3306 and R108 Wild Type under Effect of Adding 0.1&10 µm NPA

Table 2: The T Test for NPA Exp. at a Level of 5% Probability. The Test of Comparison of Averages Was Not Applied Because the F of Interaction Was Not Significant Differ Between Themselves. GA = 2.73333

VC% = 23.26 Midpoint = 3.00000

	1µMNPA			10µMNPA		
	4 days	**8 days**	**12 days**	**4 days**	**8 days**	**12 days**
R108	2.4000	3.3000	4.2000	2.0000	2.7000	3.4000
NF2262	2.0000	2.5000	3.2000	1.8000	2.7000	3.5000
NF3306	1.8000	2.9000	3.7000	1.6000	2.4000	3.1000

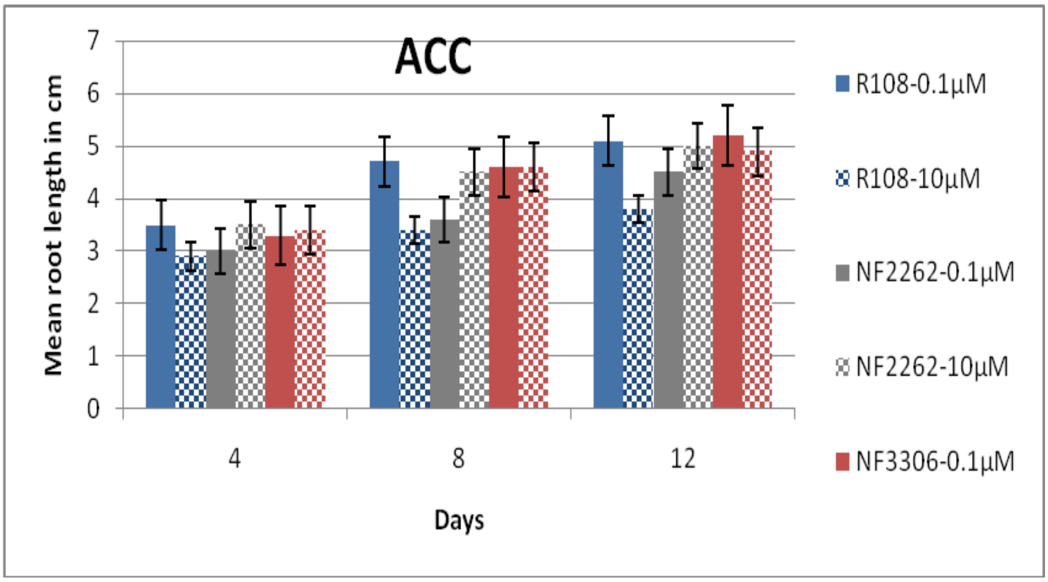

Figure 6: Comparison of Root Growth Rate between the Two Mutant Lines NF2262 & NF3306 and R108 Wild Type under Effect of Adding 0.1&10 µm ACC

Table 3: The T Test for ACC Exp. at A Level of 5% of Probability, Smd for Columns = 0.6827 Smd for Rows = 0.6827 GA = 4.08556 VC% = 13.25 Midpoint = 4.25000

	1µMACC			10µMACC		
	4 Days	8 Days	12 Days	4 Days	8 Days	12 Days
R108	3.5000 aBC	4.7000 aA	5.1000 aA	2.9000 aC	3.4000 bBC	3.8400 bB
NF2262	3.0000 aB	3.6000 bB	4.7000 aA	3.4000 aB	4.5000 aA	4.9000 aA
NF3306	3.3000 aB	4.6000 aA	5.2000 aA	3.4000 aB	4.5000 aA	4.9000 aA

DISCUSSIONS

Novel *sunn* alleles were identified and the mutated loci were precisely mapped. *Tnt1-* insertion was mapped at 462 bp and 406 bp upstream ATG start codon in NF3306 and NF2262 respectively within *SUNN* gene. The two new independent mutant alleles relay under the *nitrogen-tolerant symbiosis* group (*nts*) similar to the previously described *sunn* mutant. *SUNN* and its orthologous genes including; *HAR1*, *SYM29* and *NARK* encode receptor like kinase (RLK) protein which harbor leucin-rich repeats (LRRs), a trans-membrane domain and serine/threonine kinase domain (Schnabel et al. 2005). The nts group is shoot-regulated mutant that is defected in perception of root-derived infection signal and/or in transmission of shoot-autoregulaion signal. *sunn* is defective in perception of such signal which is in normal case perceived via LRR-RLK that encoded by *SUNN* and leads to a reduction in auxin loading from shoot root. Therefore *sunn* is lacking in long-distance auxin transport regulation by Rhizobia so, it has enlarged auxin content due to enlarged auxin transport from shoot.

phytohormones are signaling machinery that control all developmental process in plant cell through a signaling network. Final developmental phenotype is mainly depending on a complex hormonal pathway interaction. The ability of different phytohormones to affect the autoregulation mutant’s phenotype of the two new alleles was tested and compared to the wild type. Auxin is one of the major groups of endogenous phytohormones that play crucial roles in cell activity regulating and developmental progress. It is likely known that Polar Auxin Transport (PAT) is intermediated by auxin importer protein, AUX1, and auxin exporter protein, PIN while Auxin signaling is mostly intermediated by three protein families: the transcription factors which is responsible for regulation of auxin-inducible gene expression AUXIN RESPONSE FACTOR (ARF), transcriptional inhibitors auxin/indole-3-acetic acid (AUX/IAA) that interact with the ARFs and prevent their action, and F-box proteins that control the ubiquitin-mediated degradation of the AUX/IAA in response to auxin (Leyser, 2006). Consensus with the fact that auxin has a negative effect in root length regulation; it was observed that in both NF3306&NF2262 mutant alleles and wild type, the addition of slight concentration of exogenous auxin into growth media altered root growth as well as the high concentration, this results agree with Prayitno et al. 2006. The inhibitory effect of IAA prolonged concentration can be explained by either a negative feedback effect of IAA on itself with increasing exogenous addition or it has a reflecting action with ethylene signal that is negatively affecting long distance auxin transport. Results indicated that a tiny concentration of IAA has no impact on rate of root growth while the excessive concentration has a suppressive action in root elongation. We can easily detect that in case of both NF2262 and NF3306, the addition of greater concentration of IAA has less inhibitory effect than wild. This is because of positive local auxin regulation of SUNN on root region which is defected in both mutant alleles or due to the already existing massive

load of auxin from shoot to root and consequently high auxin content within root (van Noorden et al. 2006). Despite that auxin basipetal transportation in root can be inhibited by NPA, it didn't prevent auxin accumulation in elongation zone (Rashotte et al. 200). NPA obstruct with PIN proteins flanked by the endosomal vesicles and plasma membrane (Blakeslee et al. 2000).Data revealed that root elongation was inhibited by elevation concentration of auxin inhibitor in wild type as NF2262 and NF3306 *sunn* alleles. Previous results assumed that root elongation suppression ability of NPA strongly affects wild type than the mutants and NPA has more negative impact on root elongation than IAA.

Concerning the influence of exogenous ethylene precursor ACC, results indicated that the wild type root length is strongly inhibited by ACC while the two mutant alleles (NF2262&3306) showed normally root elongation taking their short root phenotype in account. In wild type treated with ACC, ethylene motivates both auxin biosynthesis and auxin basipetal transport toward the elongation zone and where it activates a local auxin response leading to inhibited cell elongation. In addition, ethylene modulates the transcription of several components of the auxin transport machinery. In contrary, in *sunn* which is defected in auxin perception, ethylene cannot trigger the auxin response nor control root growth. Previous physiological analyses is distinguished the nodulation phenotype of *sunn* mutant from that of the other hyper-nodulating *sickle* mutant; *sickle* is altered in perception of ethylene while *sunn* showed a normally sensitivity to ethylene. Instead, *sunn* displays insensitivity root growth to ethylene (Penmetsa et al. 2003).Carroll and Wopereis descried equivalent results of super-nodulation mutants of *G. max* and *L. japonicas* respectively to those of *sunn* (Carrollet al. 1985; Wopereiset al. 2000). On the other hand Penmetsa suggested that *sunn* mutant is sensitive to ethylene even if the root elongation process is insensitive (Penmetsa et al. 2003). His hypothesis was depending on the facts that root diameter and root hair density increasing with ACC treatments. The other hypothesis was described by Stepanova, he supposed that root growth inhibition by ethylene is arbitrated by ethylene-induced auxin signal and if auxin levels are already high in *sunn*, thus ethylene might not increase auxin levels more than it is enlarged, or the increasing level in auxin might not further inhibit root growth than it is reduced (Stepanova et al. 2005).Overall indicates that the complexity interplay among diverse phytohormones requires more investigation and analysis.

CONCLUSIONS

Two mutant alleles of sunn are identified that contain tobacco retrotransposon *Tnt1*-mobile element. *Tnt1*-insertion is located at 462 bp and 406 bp upstream of ATG start codon in NF3306 and NF2262 respectively within *SUNN* gene. The hyper-nodulating phenotype is resulting from defective in the pathway of complex hormonal interaction.

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